LY-294002 [2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one] Affects Calcium Signaling in Airway Smooth Muscle Cells Independently of Phosphoinositide 3-Kinase Inhibition

Barbara Tolloczko, Petra Turkewitsch, Mustafa Al-Chalabi, and James G. Martin

Meakins-Christie Laboratories, McGill University, Montreal, Quebec, Canada

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ABSTRACT

Phosphoinositide 3-kinase (PI3K) may potentially influence intracellular [Ca^{2+}], concentration by several mechanisms. We have investigated the effects of phosphoinositide 3-kinase (PI3K) inhibitors wortmannin and LY-294002 [2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one] on Ca^{2+} signaling in rat airway smooth muscle (ASM) cells using fura-2 and imaging methodology. Wortmannin (1 μM) and LY-294002 (1 and 10 μM) had opposite effects: wortmannin caused a small increase, whereas LY-294002 caused a significant decrease of peak Ca^{2+} responses to serotonin (5-HT). LY-294002 (10 μM) diminished 5-HT-induced ASM cell contraction, measured as a change in cell surface area, and inositol phosphate formation, measured by anion exchange chromatography. Thin layer chromatography revealed that the levels of phospholipase C (PLC) substrate phosphatidylinositol 4,5-bisphosphate were not affected. SDS polyacrylamide gel electrophoresis and Western blotting have shown that both wortmannin and LY-294002 inhibited platelet-derived growth factor-induced PI3K activation. However, PI3K activation could not be detected after 5-HT stimulation. The specific casein kinase-2 (CK2) inhibitor 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole (10–40 μM) reduced 5-HT-triggered responses to a similar extent as LY-294002. We conclude that LY-294002 modulates Ca^{2+} signaling in rat ASM independently of its action on PI3K by acting on, or upstream of, PLC, possibly by inhibiting CK2.

It is now generally recognized that phosphoinositides play an important role in intracellular signaling not only as enzyme substrates but also as site-specific signals that recruit and regulate protein complexes at a membrane/cytoskeletal interface. Of particular interest are phosphoinositides phosphorylated in the D-3 position, especially phosphatidylinositol (3,4,5)-trisphosphate [PI(3,4,5)P_3], which was shown to modulate a variety of cellular functions including cell proliferation, chemotaxis, and cytoskeletal remodeling. Wortmannin and LY-294002 have an inhibitory effect on contraction of several types of SM (Ibitayo et al., 1998; Kawabata et al., 2000; Yang et al., 2000; Northcott et al., 2002). It has been also reported that in vascular and colonic SM, several contractile agonists activate PI3K (Saward and Zahradka, 1997; Su et al., 1999; Takahashi et al., 1999). Contractile agonists induce Ca^{2+} mobilization by activating phospholipase C (PLC), production of 1,4,5-inositol trisphosphate, and release of Ca^{2+} from intracellular stores, followed by Ca^{2+} influx from the extracellular milieu. PI(3,4,5)P_3 may affect Ca^{2+} release by increasing activity of the gamma isoform of PLC (PLCγ) either directly through binding to the pleckstrin homology domain (Falasca et al., 1998) and the Src homology (SH2) domain (Rameh et al., 1998), or indirectly after activating Bruton's kinase Btk (Li et al., 1997). PI(3,4,5)P_3 has been also implicated in the activation of small GTPase proteins from the Rho family, which in turn stimulate production of phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2] through activation of PI(4,5)-kinase (Smith and Chang, 1989; Hartwig et al., 1995; Smith and Chang, 1990).

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ABBREVIATIONS: PI(3,4,5)P_3, phosphatidylinositol (3,4,5)-trisphosphate; PI3K, phosphoinositide 3-kinase; LY-294002, (4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; ASM, airway smooth muscle; SM, smooth muscle; PLC, phospholipase C; PI(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; 5-HT, 5-hydroxytryptamine; IP, inositol phosphate; PI, phosphoinositide; CK2, casein kinase-2; DRB, 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole; FBS, fetal bovine serum; PDGF, platelet-derived growth factor; PDGF BB, platelet-derived growth factor B subunit homodimer; KHB, Krebs-Henseleit buffer.
We have previously shown that in cultured rat ASM cells 5-HT-triggered Ca\(^{2+}\) transients involve neither PLC\(\gamma\) (Tolloczko et al., 2000) nor L-type channels (Tolloczko et al., 1995) but are dependent on PI(4,5)P\(_2\) levels (Tolloczko et al., 2002). Therefore, we have chosen this experimental system to test whether the PI3K inhibitors LY-294002 and wortmannin affect 5-HT-evoked Ca\(^{2+}\) release by modulating phosphoinositide turnover. Surprisingly, only LY-294002 significantly affected [Ca\(^{2+}\)]\(i\), indicating that PI3K is probably not involved in modulating of 5-HT-induced Ca\(^{2+}\) transients. To determine which steps of signaling were affected by LY-294002, we examined its effects on 5-HT-induced ASM contraction and inositol phosphate (IP) as well as phosphoinositide (PI) turnover. To test whether LY-294002 may exert its effects through an action on casein kinase 2 (CK2), we examined the effects of specific CK2 inhibitor 5,6-dichloro-1-\(\beta\)-ribofuranosyl-benzimidazole (DRB) on 5-HT-induced Ca\(^{2+}\) mobilization.

### Materials and Methods

#### Cell Culture

Tracheal smooth muscle cells from 7- to 9-week-old male Fisher rats (Harlan, Walkerville, MD) were isolated and cultured according to previously described methods (Tolloczko et al., 1995). Briefly, the cells were enzymatically dissociated with 0.05% elastase type IV and 0.2% collagenase type IV and cultured in 1:1 Dulbecco’s modified Eagle’s medium-Ham’s F-12 medium supplemented with 10% fetal bovine serum (FBS), 0.224% NaHCO\(_3\), and 1% penicillin/streptomycin in the presence of 5% CO\(_2\). Cell culture reagents were purchased from Invitrogen Canada (Mississauga, ON, Canada). First or second passage cells were rendered quiescent by incubation in medium containing 0.5% FBS for 4 days before experiments. Confirmation of a smooth muscle phenotype was based on typical morphology, positive smooth muscle-specific \(\alpha\)-actin staining, negative keratin staining, and contractile responses to agonists.

#### Ca\(^{2+}\) Measurements

Cells were loaded with the Ca\(^{2+}\)-sensitive dye fura-2AM (Molecular Probes, Eugene, OR) according to the previously described methods (Tolloczko et al., 2002) and imaged using an intensified charge-coupled device camera (IC200) and PTI (Photon Technology International, Princeton, NJ) software at a single emission wavelength (510 nm) with a double excitation wavelength (340 and 380 nm). Fluorescence ratio (340/380) was measured in cells stimulated with 5-HT after treatment with LY-294002 (BIOMOL Research Laboratories, Plymouth Meeting, PA) at concentrations of 1 and 10 \(\mu\)M, wortmannin (Sigma-Aldrich, St. Louis, MO) at a concentration of 1 \(\mu\)M, DRB at a concentration of 10, 20, or 40 \(\mu\)M (BIOMOL Research Laboratories), or an appropriate vehicle. [Ca\(^{2+}\)]\(i\), was calculated according to the formula of Grynkiewicz et al. (1985).

#### SDS-Polyacrylamide Gel Electrophoresis and Western Blotting

Electrophoresis reagents were obtained from Bio-Rad (Mississauga, ON, Canada). Populations of cells stimulated with 1 \(\mu\)M 5-HT or 100 ng/ml PDGF BB, with or without LY-294002 and wortmannin preincubation, were rinsed with ice-cold PBS, lysed with 1 ml of lysis buffer (50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EGTA, 1.5 mM MgCl\(_2\) 1 mM phenylmethylsulfonfluoride, 20 \(\mu\)M leupeptin, 15 U of aprotinin/ml, and 1 mM sodium orthovanadate), and clarified by centrifugation. Protein concentration was determined and equal amounts (20 \(\mu\)g) resolved by 10% SDS-PAGE, transferred onto 0.22-\(\mu\)m pore nitrocellulose filters and probed with monoclonal antibodies that recognized either activated protein kinase B/Akt or total Akt (Cell Signaling Technology Inc., Beverly, MA). Proteins were visualized using a secondary antibody conjugated to horseradish peroxidase and enhanced chemiluminescence (Amersham Biosciences Inc., Oakville, ON, Canada) on a FluorChem 8000 imaging system (Alpha Innotech Corp.).

#### Measurement of Inositol Phosphates

Confluent cells plated onto 60-mm-diameter dishes were grown arrested and radiolabeled for 48 h with inositol-free Dulbecco’s modified Eagle’s medium containing 0.5% FBS and 1 \(\mu\)Ci/ml [\(^{3}H\)myo-inositol (Amersham Biosciences Inc.). Cells were washed free of unincorporated [\(^{3}H\)myo-inositol with Krebs-Henseleit buffer (KHB; 117 mM NaCl, 4.7 mM KCl, 1.1 mM MgSO\(_4\), 1.2 mM KH\(_2\)PO\(_4\), 20 mM NaHCO\(_3\), 2.4 mM CaCl\(_2\), 1 mM glucose, and 20 mM HEPES) and preincubated with 10 mM LiCl in 1 ml of PBS at 37°C for 10 min with or without 10 \(\mu\)M LY-294002. The cells were challenged with 5-HT in the presence of LiCl for 10 min at 37°C. Stimulation was terminated by the addition of ice-cold 3 M trichloroacetic acid. Cells were chilled on ice for 30 min and then scraped off the dishes. After sonication, cellular debris was removed by centrifugation at 4000g for 5 min at 4°C. The inositol polyphosphates were extracted with 10 mM EDTA/trichlorotrifluoroethane/tri-n-octylamine (1:2:2), and the aqueous and organic fractions were separated by centrifugation. The aqueous layer was neutralized with 60 mM NaHCO\(_3\) and assayed for total [\(^{3}H\)myo-inositol phosphates by anion exchange chromatography. Samples were loaded onto columns containing 1 ml of the formate form of Dowex 1-X8 resin (Bio-Rad) and [\(^{3}H\)myo-inositol and [\(^{3}H\)glycerophosphoinositides were removed by washing with 60 mM ammonium formate and 5 mM sodium tetraborate. [\(^{3}H\)myo-inositol phosphates were eluted with 1 M ammonium formate and 100 mM formic acid. Five independent experiments were conducted, each with duplicate samples. Data are expressed as percentage of control.

#### Phosphoinositide Analysis

Confused cells cultured on 150-mm dishes were labeled with 2.5 \(\mu\)Ci/ml [\(^{3}H\)myo-inositol in inositol-free Dulbecco’s modified Eagle’s medium containing 0.5% FBS for 48 h. Cells were washed with KHB, incubated with 10 \(\mu\)M LY-294002 or the vehicle for 10 min, followed by 10-min stimulation with 1 \(\mu\)M 5-HT or KHB. The cells were washed twice with ice-cold PBS and scraped from the plates in 1 ml of PBS. To extract lipids, cells were centrifuged at 10,000g for 5 min, and the cell pellet was resuspended in 400 \(\mu\)l of Triton extraction buffer (25 mM HEPES, pH 7.2, 250 mM NaCl, 2 mM MgCl\(_2\), 2 mM MnCl\(_2\), 1 mM CaCl\(_2\), and 0.5% Triton X-100) and incubated for 20 min at 4°C with mixing as described previously (Speed and Mitchell, 2000). Lysates were centrifuged at 15,000g for 10 min, and the supernatant was collected as the Triton-soluble fraction to which 400 \(\mu\)l of stop solution [methanol/concentrated HCl, 10:1 (v/v)] was added. The Triton-insoluble pellet was washed twice with PBS, and 200 \(\mu\)l of stop solution and 200 \(\mu\)l of PBS were added. Lipids from Triton-soluble and Triton-insoluble fractions were extracted using chloroform/methanol [1:1 (v/v)]. Samples were centrifuged at 15,000g for 2 min at 4°C, and the lower phase containing phosphoinositides was collected. After reduction of the solvent volume of the sample to approximately 100 \(\mu\)l under vacuum, the samples were applied with a glass capillary onto potassium oxalate impregnated silica gel thin layer chromatography plates. Potassium oxalate plates were prepared by placing silica gel
plates (Analtech, Newark, DE) in a 1% potassium oxalate solution in methanol/water (60:40 (v/v)) for approximately 30 min. After drying in the fume hood for about 1 h, the plates were dried in a 110°C oven for 15 to 20 min, and stored in a low humidity cabinet. The plates were developed in chloroform/acetone/methanol/acetic acid/water [40:15:13:12:8 (v/v/v/v/v)]. Chromatographed lipids were localized by iodine staining and identified by migration with authentic standards: PI, phosphatidylinositol 4-phosphate, and PI(4,5)P2. Lipids were scraped from the plates, and 3H radioactivity was measured using a scintillation counter. Four independent experiments were conducted and the amount of phosphatidylinositol bisphosphate is expressed as a percentage of the total counts.

**Measurement of Cell Contraction.** Cells used for contraction measurements were plated on laminin-coated (0.01 mg/ml) microscopic coverslips and rendered quiescent by serum deprivation at approximately 70% confluence. The cells were incubated for 10 min with 10 μM LY-294002 or with the appropriate vehicle. Images of cells stimulated with 1 μM 5-HT or Hanks’ buffer were acquired at the rate of one image per minute using a videocamera (Hamamatsu Photonics, Hamamatsu City, Japan) mounted on a microscope equipped with Nomarski optics (Nikon Diaphot; Nikon Corp., Tokyo, Japan) and PTI software. The surface area of the cells was measured before and 10 min after stimulation. Three experiments were performed with 29 to 36 cells recorded in each experiment, and data are expressed as percentage of cell area decrease. Negative values indicate increase in cell area.

**Statistical Analysis.** Statistical analysis was performed using Kolmogorov-Smirnoff test for contraction measurements and unpaired Student t test for remaining experiments. p values ≤0.05 were considered as statistically significant.

**Results**

**Effect of LY-294002 and Wortmannin on 5-HT-Induced Ca2+ Release.** Cultured rat tracheal smooth muscle cells were incubated for 10 min with LY-294002 at concentrations of 1 or 10 μM or wortmannin at a concentration of 1 μM or an appropriate vehicle and 5-HT-evoked Ca2+ transients were measured. Peak Ca2+ responses, corresponding to the release of Ca2+ from intracellular stores (Tolloczko et al., 1995), were significantly lower in the cells preincubated with 1 and 10 μM LY-294002 (253.56 ± 8.93 and 171.55 ± 12.27 nM, respectively, compared with 380.93 ± 14.14 nM in controls; p < 0.05) (Fig. 1a). In the cells preincubated with wortmannin, 5-HT-triggered Ca2+ responses were higher than in controls (474 ± 19.22 and 378.23 ± 15.46 nM, respectively (Fig. 1b). These results indicate that two inhibitors described as specific for PI3K have opposite effects on 5-HT-induced Ca2+ mobilization in ASM cells.

**Effect of LY-294002, Wortmannin, and 5-HT on PI3K Activation.** To confirm that LY-294002 and wortmannin...
inhibit PI3K activation at the concentrations used in the study of agonist induced Ca\(^{2+}\) transients, ASM cells were preincubated with 10 \(\mu\text{M}\) LY-294002, 1 \(\mu\text{M}\) wortmannin, or an appropriate vehicle and were stimulated with 100 ng/ml PDGF BB, as a positive control. Lysates of these cells were tested for the presence of activated Akt, the downstream target of PI3K. Wortmannin (1 \(\mu\text{M}\)) completely inhibited Akt phosphorylation, whereas 10 \(\mu\text{M}\) LY-294002 caused an approximately 50% decrease in Akt activation (Fig. 2a) consistent with the reports indicating that wortmannin is a more potent inhibitor of PI3K than LY-290042 and that the IC\(_{50}\) of LY-294002 is 10 \(\mu\text{M}\) (Davies et al., 2000). To determine whether the effects of LY-294002 on 5-HT-induced [Ca\(^{2+}\)]\(\text{i}\) increase result from its effects on PI3K activity, Akt phosphorylation was assessed in lysates from cells stimulated with 5-HT for different periods of time. No Akt activation could be detected for up to 10 min of stimulation with 5-HT, whereas PDGF BB caused a robust response (Fig. 2b). Immunoprecipitation with anti-Akt antibodies followed by immunoblotting with phosphospecific anti-Akt antibodies also failed to detect Akt activation in 5-HT stimulated cells (data not shown).

**Effect of LY-294002 on 5-HT-Induced IP Production.** To establish whether the inhibitory effect of LY-294002 on Ca\(^{2+}\) mobilization is exerted downstream or upstream of PLC, 5-HT-induced IP production was measured in control and LY-294002-treated cells. LY-294002 (10 \(\mu\text{M}\)) caused a significant decrease in IP turnover in cells stimulated with 1 \(\mu\text{M}\) 5-HT (176.29 \pm 35.37% of baseline, compared with 300.48 \pm 48.68% in controls; \(p = 0.05\)) without significant effect on the turnover in unstimulated cells (92.64 \pm 23.34% of baseline; Fig. 3), suggesting that LY-294002 affects agonist-induced Ca\(^{2+}\) responses at, or upstream, of PLC.

**Effect of LY-294002 on PI(4,5)P\(_2\) Pool.** To verify whether LY-294002 affects levels of the PLC substrate, PI(4,5)P\(_2\), lipids were extracted from unstimulated and 5-HT-stimulated cells preincubated with LY-294002 or the vehicle, and the level of PI(4,5)P\(_2\) was assessed by thin layer chromatography. Because changes in PIs may be spatially restricted (Hope and Pike, 1996), we analyzed the effects of LY-294002 on PIs in Triton-insoluble fraction, representing caveolae and/or lipid rafts and therefore the agonist accessible pool (Pike and Casey, 1996), and Triton-soluble fraction containing lipids from other regions. LY-294002 had no significant effect on PI(4,5)P\(_2\) levels either in unstimulated cells or in 5-HT-stimulated cells. PI(4,5)P\(_2\) accounted for 7.80 \pm 1.04% of total radioactivity in control cells and 9.40 \pm 0.30% in LY-294002-treated cells in Triton-soluble fraction, and for 2.12 \pm 0.51 and 3.12 \pm 1.33%, respectively, in Triton-insolubl...
uble fraction. In 5-HT-stimulated cells, PI(4,5)P₂ constituted 9.00 ± 0.54 in control cells and 9.06 ± 0.97 in LY-294002-treated cells in the Triton-soluble fraction and 4.69 ± 1.94 and 3.25 ± 1.28% (p, N.S.), respectively, in the Triton-insoluble fraction (Fig. 4). These data indicate that LY-294002 does not affect baseline or agonist induced PLC substrate turnover.

Effect of LY-294002 on ASM Cell Contraction. To confirm that LY-294002-induced modulation of Ca²⁺ transients also affects SM contraction, cells were incubated for 10 min with 10 μM LY-294002 and stimulated with 1 μM 5-HT. LY-294002 caused a significant (p = 0.001) decrease in 5-HT-evoked cell contraction affecting both the number of contracting cells and a degree to which they contracted (Fig. 5). To assess the contribution of a nonspecific stimulus, cell area was measured in control and LY-294002-treated cells before and after addition of Hank's buffer alone. Hank's buffer caused a small (0.54 ± 0.48%) decrease in cell area that was not significantly affected by LY-294002 (0.1 ± 0.51% increase in cell area) (Fig. 5, inset). These results show that LY-294002 inhibits 5-HT-induced contraction in ASM cells.

Effect of DRB on 5-HT-Induced Ca²⁺ Release. To test whether the effect of LY-294002 on Ca²⁺ signaling is due to the inhibition of CK2 kinase, cells were incubated for 10 min with CK2 inhibitor DRB at the concentration of 10, 20, or 40 μM and stimulated with 1 μM 5-HT in the presence of the inhibitor. DRB significantly reduced Ca²⁺ mobilization in a dose-dependent manner. Peak Ca²⁺ responses were 263.30 ± 8.44 nM in cells treated with 10 μM DRB, 219.48 ± 4.90 nM in 20 μM DRB, and 191.33 ± 7.24 nM in 40 μM DRB, compared with 321.30 ± 7.39 nM in controls (Fig. 6).

Discussion

Our study has shown that LY-294002, widely used as a specific inhibitor of PI3K, blunted Ca²⁺ responses of ASM cells to 5-HT. However, wortmannin, another PI3K inhibitor structurally unrelated to LY-294002, caused an increase of Ca²⁺ transients. Moreover, we did not observe 5-HT stimulated phosphorylation of downstream substrate of PI3K, Akt. Akt phosphorylation and activation have been described as being universally induced on PI3K activation (Coffer et al.,...
Our observation is consistent with previous reports showing that 5-HT failed to activate PI3K and cause Akt phosphorylation in bovine carotid artery (Komalavilas et al., 2001) and in rat aortic SM cells (Banes et al., 1999). However, endothelin and angiotensin, which act through similar G protein-coupled mechanisms did activate PI3K in intestinal and vascular smooth muscle, respectively (Saward and Zahradka, 1997; Su et al., 1999), showing that PI3K activation downstream of G protein-coupled receptors may be tissue or receptor specific.

LY-294002 may also affect vascular SM contractility by decreasing baseline levels of PI3K activity (Saward and Zahradka, 1997). However, we were not able to detect baseline Akt phosphorylation in our experiments. This result is supported by the observation that baseline PI3K activity in cultured vascular SM cells was much lower than in SM strips (Saward and Zahradka, 1997). We validated the tested concentrations of LY-294002 and wortmannin by examining their ability to reduce PDGF-stimulated activation/phosphorylation of Akt. The inhibitory effect of wortmannin on PDGF-activated Akt was more pronounced than the effect of LY-29402. These data indicate that LY-294002 may have an effect on Ca\(^{2+}\)-signaling unrelated to its effect on PI3K. Further experiments indicated that decreased Ca\(^{2+}\) release resulted in decreased cell contraction and that LY-294002 affected Ca\(^{2+}\) signaling at, or upstream of, PLC level.

There are very few published data that would allow us to speculate about possible mechanisms of this effect. LY-294002-induced inhibition of 5-HT-triggered contraction in rat aorta was attributed to LY-294002 being a 5-HT receptor antagonist (Komalavilas et al., 2001). It is possible then that it can have similar effect on rat ASM cells. It has been also demonstrated that LY-294002 inhibits a pleiotropic, constitutively active serine/threonine CK2 with a potency similar to that for PI3K (Davies et al., 2000). Our preliminary results indicate that the CK2 inhibitor DB-5 affects 5-HT-induced Ca\(^{2+}\) signaling in a similar manner to LY-294002. The role of CK2 in Ca\(^{2+}\) signaling is not known. It is interesting, however, that catalytic activity of CK2 increases upon tyrosine phosphorylation by kinases from the Src family (Donella-Deana et al., 2001) and in rat ASM cells 5-HT stimulation causes a Ca\(^{2+}\) transient that is partially dependent on Src activation (Tolloczko et al., 2002) The effect of Src inhibition is exerted, similar to the effect of LY-294002, at the PLC level, or upstream of it, without affecting PLC\(\gamma\). Although we have previously shown that Src modulates the level of PI(4,5)\(P_2\) in the agonist accessible lipid fraction, it is conceivable that Src may have multiple effects, including CK2 activation and PI(4,5)\(P_2\) regulation. CK2 may also phosphorylate G\(\alpha\) interacting protein, a member of the RGS family (regulators of G protein signaling) (Fischer et al., 2000) and therefore potentially affects signaling by 5-HT that acts on G protein-coupled receptors.

We conclude that inhibition of SM contraction by LY-294002 may result, at least partially, from its effects on Ca\(^{2+}\) signaling independently of its effects on PI3K, possibly involving CK2. Further studies are needed to establish the detailed mechanisms underlying this phenomenon.

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**Address correspondence to:** Dr. James G. Martin, Meakins-Christie Laboratories, McGill University, 3626 St-Urbain St., Montreal, Quebec, Canada H2 2P2. E-mail: james.martin@mcgill.ca